Expression and Isolation of Recombinant Microneme 3 (MIC3) Protein of *Toxoplasma gondii* Local Isolate on *Eschericia coli* (BL21)

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Abstract. Toxoplasmosis is a disease that infects all warm-blooded animals, including livestocks and humans caused by *Toxoplasma gondii* parasites. There are major drugs used for the therapy, though they have some effects to the patients, such as allergy, toxic and teratogenic for fetus. In addition, toxoplasmosis treatment is only effective for tachyzoites *T. gondii* in acute infection, while tissue cysts cannot be eradicated in chronic toxoplasmosis Tissue cysts of *T. gondii* contained in meat that are consumed by humans and meat-derived products may be important sources of infection for humans. Microneme protein (MIC) is one of proteins that belongs to excretory-secretory antigens (ESAs) of *Toxoplasma gondii*. Microneme 3 protein (MIC3) is the protein that plays an important role in the invasion process during cell infection as a mediator attachment parasite to the host cell. Recombinant MIC3 protein has been already used for the detection of toxoplasmosis and it could induce humoral and cellular immune response in experimental animals. The aim of this research was to express MIC3 recombinant protein of *T. gondii* from local isolate that was cloned into expression vector and transformed to *E. coli* BL21. In the future, recombinant protein MIC3 can be used for vaccine candidate and diagnostic tools for toxoplasmosis in animals and humans. Gene of MIC3 *T. gondii* local isolate (1.2 Kbp) was cloned into expression vector pET-32a(+) (5.9 Kbp) and transformed to *Escherichia coli* BL21. Protein from plasmid recombinant (7.1 Kbp) was expressed and performed by culturing recombinant bacteria into LB medium containing ampicillin and IPTG. Recombinant protein was isolated by sonication method and identified using SDS-PAGE. Finally, the recombinant protein was analyzed by immunoblotting using anti-ESAs polyclonal antibody. In conclusion, expression of the MIC3 gene with ~108 kDa has been successfully performed by cloning gene encoding for MIC3 protein of *T*. gondii local isolate that can be identified with polyclonal antibody anti-ESAs.

Keywords: *Toxoplasma gondii*, expression, MIC3 protein

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects all warm-blooded animals, including livestocks and humans, causing toxoplasmosis (Black and Boothroyd, 2000). All mammals and birds that are consumed by humans may serve as intermediate hosts for *T. gondii* and thus, may be a potential source of infection for humans (Tenter, 2009).

In animals, toxoplasmosis has negative impacts in the economics aspect since it can cause abortions, still birth and neonatal lost. In addition, the tissue cysts *T. gondii* in meat of infected livestock are important source of infection for humans. In pregnant woman, congenital infection can lead to miscarriage, neonates malformation or defect occurring during the development of the fetus, such as blindness (Gandahusada, 1995; Black and Boothroyd, 2000; Tenter et al., 2000; Dubey, 2007).

The life cycle of *T. gondii* includes asexual multiplication in various tissues of intermediate hosts and sexual reproduction in the intestine of definitive hosts. Intermediate hosts are probably all warm-blooded animals including most livestocks and humans. Definitive hosts are members of the family Felidae, for example domestic cats. Tissue cysts of *T. gondii*

contained in meat and meat-derived products may be important sources of infection for humans. In livestock, *T. gondii* tissue cysts are most frequently observed in various tissues of infected pigs, sheep and goats, and less frequently observed in infected poultry, rabbits, dogs and horses. By contrast, tissue cysts are found only rarely in skeletal muscles of cattle or buffaloes (Tenter, 2009).

There are major drugs used for the therapy of toxoplasmosis, such as sulfonamide and pyrimethamine which are usually effective for therapy. Nevertheless, these drugs have some effects to the patients, such as allergy, toxic and especially for pregnant women, pyrimethamine could cause teratogenic for the fetus. Moreover, toxoplasmosis treatment is only effective for tachyzoites *T. gondii* in acute infection, while bradyzoites or tissue cysts cannot be eradicated in chronic toxoplasmosis (Gandahusada, 1995; Carruthers, 2004). Therefore, the studies about prevention and treatment of toxoplasmosis are important to conduct.

In molecular terms, invasion of host cells by *T. gondii* is closely coupled by the release of proteins stored within apical secretory granule knows as micronemes, which play a central role in the recognition of and adhesion to the hosts cells, rhoptries and dense granules (Ismael et al., 2003; Beghetto et al., 2005; Buffolano et al., 2005). Among these proteins, the MIC3 protein contains adhesive motifs and has been shown to bind to the surface of the host cells. There are Lectin-like domain required for binding and Epidermal Growth Factor-like (EGF-like) domain containing human B and T cell epitopes (Garcia-Réquet et al., 2000; Cérède et al., 2002; Beghetto et al., 2005).

Several previous studies have demonstrated the usefulness of recombinant antigens for serological diagnosis of *T. gondii*, such as SAG (surface antigen) (Beghetto et al., 2005; Buffolano et al., 2005), GRA (Igarashi et al., 2008; Wihadmadyatami, 2009), ROP (Ahn et al., 2006; Igarashi et al., 2008) and MIC (Ismael et al., 2003; Beghetto et al., 2005; Buffolano et al., 2005). The use of recombinant antigens would allow better standardization of the tests; reduce production costs, reproducible, safer and efficient to produce recombinant protein in adequate amount (Hiszczyńska-Sawicka, 2005). The result study of MIC3 (Ismael et al., 2003; 2009) shows that cloning and expression a recombinant plasmid DNA encoding the MIC3 of *T. gondii* RH isolate induced a strong specific humoral and cellular immune responses. This study was the first demonstration showing that the MIC3 of *T. gondii* is a potent and effective vaccine candidate against toxoplasmosis.

The study was aimed to express recombinant MIC3 gene encoding MIC3 protein that has been successfully cloned into *E. coli* (Indrasanti et al, 2011) to produce MIC3 recombinant protein. It can be used as vaccine candidate and toxoplasmosis diagnostic tool for animals especially livestocks, and humans.

Materials and Methods

Toxoplasma gondii tachyzoites DNA and Female Balb/C mice, 6 to 8 weeks old, were obtained from Study Program of Biotechnology, University of Gadjah Mada, Yogyakarta. Recombinant bacterial colonies XL-1 Blue brought recombinant plasmid pGEM-T with insert MIC3 (pWTA-M3), and primer specific for MIC3 M3F1 (has *Hind*III restriction site) and M3R1 (has *Eco*RV restriction site) for positive controls (Dewi, 2006). Protein of recombinant MIC3s was recombinant protein from cloning MIC3 gene amplified using M3F1 and M3R1 primers into expression vector and transformated to *E. coli* BL21 as negative control for immunoblotting.

Isolation and cloning of MIC3 gene

Toxoplasma gondii tachyzoites DNA were amplified by Polymerase Chain Reaction (PCR) to amplify only the sequence coding for the part of MIC3 gene with *Eco*RV and *Hind*III

restriction sites at 5' end of forward and reverse primers (Invitrogene), respectively. Forward primer (M3F3): 5'GTGTGATATCCTTGTCCAACACTGGGTA3' and reverse primer (M3R3): 5'CACGAAGCTTTGCGAATGGGCG3'. Polymerase Chain Reaction product was purified by EZ-10 spin coloumn purification kit (Bio Basic Inc.), cut with endonuclease restriction enzyme EcoRV and HindIII and ligated into expression vector pET-32a(+) (Novagen). Recombinant plasmid pET-32a(+)-MIC3*a* was transformed into competent *E. coli* strain BL21 (Invitrogen).

Gene expression

Escherichia coli strain BL21 transformed with recombinant plasmid was grown in Luria Bertani (LB) medium supplemented with 25 µl/ ml ampicillin (Boehringer Mannheim) at 37 ^{o}C overnight. Five flasks containing 25 ml of LB containing 25 µl/ ml ampicillin were inoculated by 2.5 ml samples of the overnight cultures. The cultures were grown at 37 ^{o}C wih vigorous shaking until the OD_{600} ~0,5-1. The cultures were induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1, 2, 3, 4 hr at 37 ^{o}C. Induced cell and control cell were pelleted by centrifuge 3000 rpm for 20 minutes at 4 °C. The pellet was washed in 25 ml of phosphate buffered saline (PBS) I pH 7 **(**145 mM NaCl, 10 mM Na_2HPO4, 10 mM NaH2PO4 and ddH_2O up to 1 L) three times and resuspended in 250 µl PBS I. The cell pellet was sonicated six times on ice in pulses of 30 seconds and centrifuged at 12.000 rpm for 10 minutes. Right molecular weight of the recombinant protein was confirmed by sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) in comparison with protein marker.

Enzyme Linked Immunosorbent Assay (ELISA)

Levels of polyclonal antibody in serum samples were determined by standards procedures. Briefly, the 96 flat-bottom wells of microtiter plate (Nunc Gene) was coated overnight at 37°C with 5 µg/ml ESA protein in 100 µl buffer coating (0,2 M Na2CO3, 0,2 M NaHCO3). The plate was washed with washing solution (0,15 M NaCl, 0.05% Triton X-100, 0.02% NaN3); nonspecific binding sites were blocked with blocking buffer (1% BSA + 0,02% NaN3 in PBS I) for 1 h at 37°C. After the plate was washed with washing solution, it was coated with 100µl mice serum (polyclonal antibody anti-ESA) with graduate dilution 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400 and incubated for 1 h at 37^{o}C. After the plate was washed, bound antibodies were detected by incubation for 1 h at 37°C with a goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma) diluted 1:3000 in incubation buffer. After the plates were washed, the bound phosphatase activity was measured with *p*-nitrophenylphosphate at 1 mg/ml. The reaction was stopped after incubating for 15 min at 37°C; the OD_{405} of sample was then read in ELISA reader.

Western-blot analysis

The samples were prepared for SDS-PAGE by adding 12.5 µl 5x sample buffer to 50 µl of the samples. They were boiled for 2 minutes and loaded into 12% polyacrilamide gel. The proteins were transferred to nitrocellulose membrane at 500 mA for ±1-2 hr and probed using anti-ESA polyclonal antibody diluted 1:100 in incubation buffer pH 7,2 (0,1 M NaCl, 50 mM NaHPO4, 50 mM Na2HPO4, 1% BSA, 0,05% Triton X-100, 0,02% NaN3, and ddH_2O up to 1 L). The bound antibodies were detected using anti-mouse immunoglobulin G (IgG)-alkaline phosphatase conjugate (Sigma) diluted 1:3000 in incubation buffer. Alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT) liquid substrate system (Sigma). Standard molecular masses (prestained SDS-PAGE standards, Sigma marker™ Wide Range) were used.

Results and Discussion

Gene MIC3 cloning of *T. gondii* into *E. coli* has been successfully done (Indrasanti et al., 2011). In the last research, forward and reverse primers were designed with EcoRV and HindIII restriction site, respectively. Restriction sites were used to ligate insert MIC3 and expression vector pET-32a(+). Recombinant plasmid pET-32a(+)-MIC3 called as pET-M3 was transformed into competent *E. coli* strain BL21 using heat shock method. The aim of this research was to express recombinant MIC3 gene of *T. gondii* in *E. coli.* The result of this research was recombinant protein MIC3 that can be used for vaccine candidate and diagnostic tools for Toxoplasmosis in the future.

The previous study about cloned gene from secretory organelles is gained from *T. gondii*, micronemes. The entire MIC3 open reading frame was amplified from tachyzoites DNA of *T. gondii* local isolate by Polymerase Chain Reaction (PCR) by Ismael et al. (2003) and Dewi (2006). They have successfully amplified MIC3 gene using these primers and obtained amplicon 1155 bp and 4200 bp, respectively.

Amplicon of 2247 bp was sequenced, including the complete cDNA sequence and additional flanking sequences by Garcia- Réquet et al**. (**2000) (GenBank Accession No. AJ132530). This showed that the MIC3 gene contained no intron. The genomic sequence matched perfectly to the cDNA. There were no typical eukaryotic TATA or CCAAT consensus promoter sequences in the 5' non-coding region. Forward and reverse primers were attached from bases 666-692 and 1812-1833, respectively. Coding region between both primers was amplified MIC3 gene as long as 1170 bp (bases 666-1833) (Garcia- Réquet et al**.,** 2000).

Excretory secretory antigens which derives from tachyzoites *T. gondii*, is used as antigen to stimulate polyclonal antibody on female mice Balb/C. Levels of polyclonal antibody in serum samples were determined by ELISA for immunoblotting as 2,894 (diluted 1:100).

Expression of recombinant MIC3 protein has been done by culturing isolate at 37 ^{o}C with vigorous shaking until the OD_{600} ~0,5-1 for protein isolation. The cultures were induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1, 2, 3, 4 hr at 37^{o}C. Measurement of protein concentration showed at 3 h and 4 h, was 8,79 µg/ µl and 10,34 µg/ µl, respectively. The result of recombinant microneme protein purification with IPTG induction for 3 hours on 37^{o}C has been done by Beghetto (2005) and it has been successfully recognized by T cell and toxoplasmosis patient antibody using ELISA.

Expression of recombinant protein on SDS-PAGE showed more thickness band of protein on molecular mass approximately ~90 kDa. The thicknesses of band protein were showed in accordance time of gradually IPTG induction (Line 3, 4, 5, 6, 7). The BL21 *E. coli* transformed with pET-M3 expressed a dimeric recombinant MIC3 protein that was secreted into the supernatant as well as being present in the cell pellets. It had an apparent molecular mass (~108 kDa) which was ~18 kDa greater than that of the native MIC3 protein (90 kDa) (Garcia- Réquet et al**.,** 2000; Cérède et al., 2005). This finding could be explained by fusion proteins of Trx•Tag (109 aa), His•Tag (6-10 aa), and S•Tag (15 aa) from expression vector pET-32a(+) (Novagen, 2001; Igarashi et al., 2008). These results are consistent with those of Ismael et al. (2003) and Cérède et al. (2002), which demonstrated that the increase in size of the recombinant protein was due to failure to cleave the MIC3 propeptide in BHK-21 cells; this was confirmed by probing the immunoblot with a specific anti-propeptida serum. These authors also showed that the presence of the propeptide inhibited the adhesive function of the MIC3 protein but that it remained antigenic, because it was well-recognized by serum from a *T. gondii*-infected mouse. To confirm the detection of specific anti-ESA

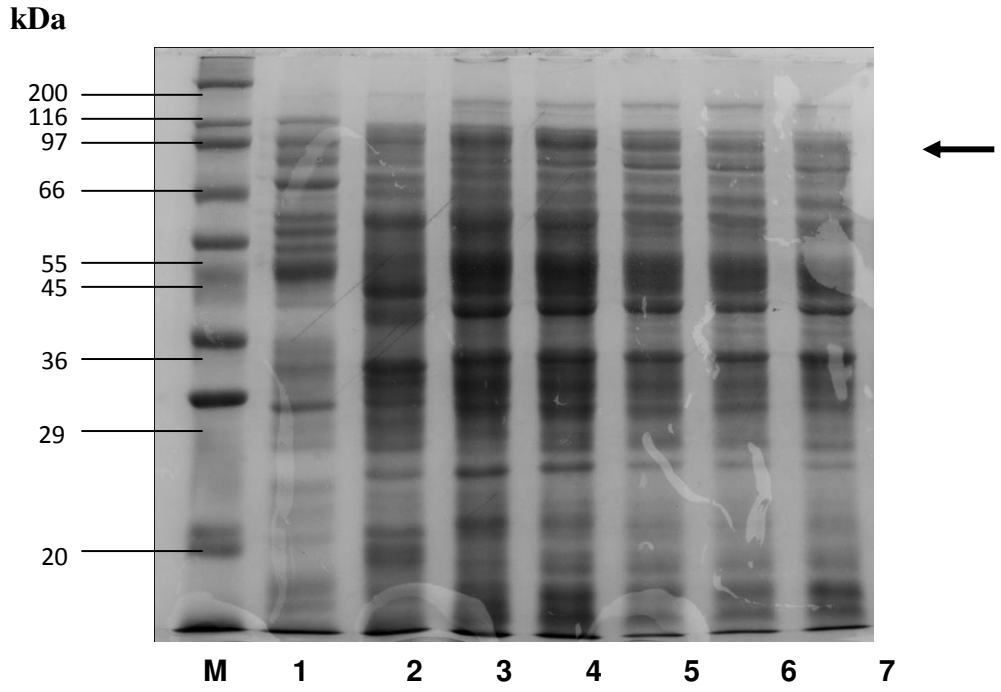

Figure 1. SDS-PAGE electrophoresis of MIC3 recombinant proteins. M, molecular mass marker (Sigma), lane 1, BL21 *E.coli* protein, lane 2, MIC3s protein, lane 3, MIC3 protein after 1 mM IPTG induction for 4 h, lane 4, MIC3 protein after 1 mM IPTG induction for 3 h, lane 5, MIC3 protein after 1 mM IPTG induction for 2 h, lane 6, MIC3 protein after 1 mM IPTG induction for 1 h, lane 7, MIC3 protein without 1 mM IPTG induction. Arrow shows band of recombinant protein.

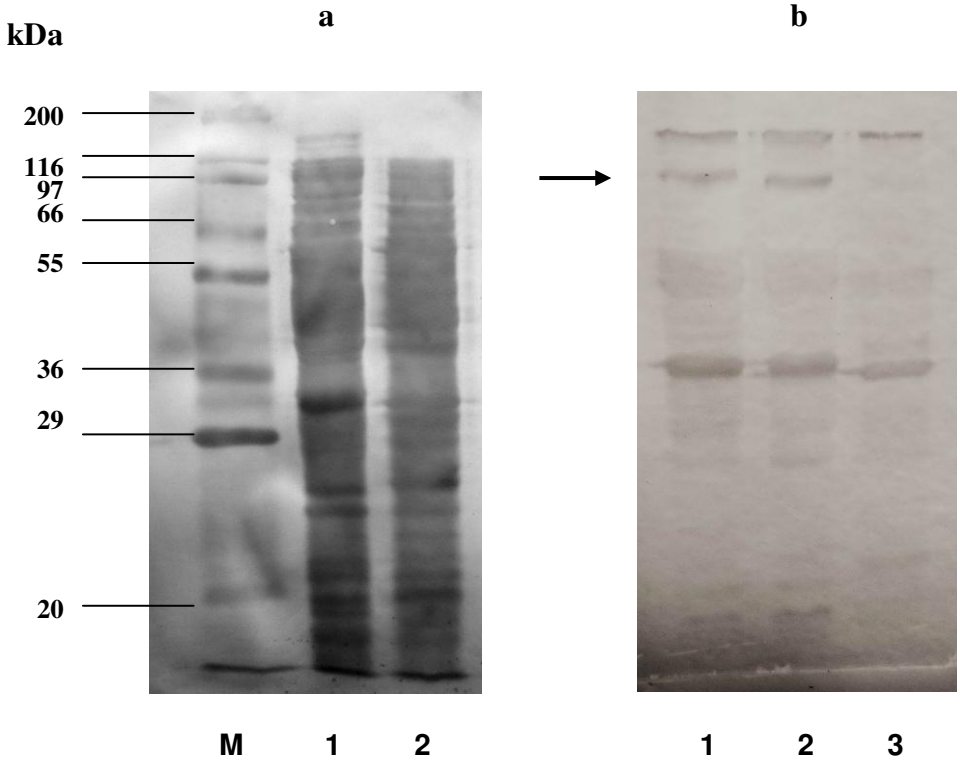

Figure 2. Western blotting using polyclonal antibody anti-ESA. Panel A: Blotting MIC3 recombinant protein prestained with amido black, M, molecular mass marker (Sigma), line 1, MIC3 recombinant protein, line 2, MIC3s recombinant protein. Panel B: Immunoblotting, line 1, MIC3 recombinant protein induced with IPTG for 4 h, line 2, MIC3 recombinant protein induced with IPTG for 3 h, line 3, protein MIC3s induced with IPTG for 4 h. Arrow shows band of MIC3 recombinant protein.

antibody by recombinant MIC-3 antigen, the Western blot was carried out.

Figure 2, Panel B shows band indicating positive reaction between polyclonal antibody anti ESA and recombinant protein pET-M3 (Line 1 and 2) whereas on recombinant protein pET-M3s, the band is not formed (negative) (Line 3).

The cross reactivity observed in the Western blotting result using polyclonal antibody against recombinant protein can be explained by the study using crude recombinant protein and polyclonal antibody, not the purified ones. The occurrence of cross-reaction should be avoided due to the mistake in analysis. Further study needs to be conducted on DNA sequence identification or amino acid sequence identification on recombinant protein. Recombinant protein purification and monoclonal antibody should be used in order to get more specific result.

Conclusion

The result of the study showed that expression of the MIC3 gene has been successfully performed with molecular weight ~108 kDa.

Acknowledgement

The authors would like to extend her gratitude to the following project personnels and consultants who have made the project a successful: Dr. Nastiti Wijayanti, S.Si., .M.Si., Mrs. Arsiah, Mr. Jono, Mr. Haryanto, Mrs. Tri, Mr. Jo, Mr. Toni, Mrs. Ika. Toxo team: Pupung, Miss Etty, Miss Yuli, Dyah, Hevi, Mrs. Sorta, Mrs. Win, Mrs. Lita.

References

Ahn HJ, S Kim, HE Kim and HW Nam. 2006. Interactions between secreted GRA proteins and host cell proteins across the parasitophorous vacuolar membrane in the parasitism of *Toxoplasma gondii.* Korean J. Parasitol. 44(4):303-312.

Beghetto E, HV Nielsen, P Del Porto, W Buffolano, S Guglietta, F Felici, E Petersen and N Gargano. 2005. A combination of antigenic regions of *Toxoplasma gondii* microneme proteins induces protective immunity against oral infection with parasite cysts. J. Infectious Disease. 191:637-645.

Black MW and JC Boothroyd. 2000. Lytic cycle of *Toxoplasma gondii*. Microbiol. and Molecular Biology Rev. 64(3):607-623.

Buffolano W, E Beghetto, M Del Pezzo, A Spadoni, M Di Cristina, E Petersen and N Gargano. 2005. Use of recombinant antigens for early postnatal diagnosis of congenital toxoplasmosis. J. Clinical Microbiol. 43(12):5916-5924.

Carruthers VB. 2004. Proteases as Potential Targets for Blocking *Toxoplasma gondii* Invasion and Replication. In: In: *Opportunistic Infections: Toxoplasma, Sacocystis, and Microsporidia*. Kluwer Academic Publishers. New York. Pp. 21-38.

Cérède O, J Dubremetz, D Bout and M Lebrun. 2002. The *Toxoplasma gondii* protein MIC3 requires pro-peptide cleavage and dimerization to function as adhesin. The EMBO J. 21(11):2526-2536.

Cérède O, J Dubremetz, M Soête, D Deslée, H Vial, D Bout and M Lebrun. 2005. Article: Synergistic role of micronema protein in *Toxoplasma gondii* virulence. J. Exp. Med. 201:1-11.

Dewi NND. 2006. Kloning Gen Penyandi Protein Mikronema 3 (MIC3) Takizoit *Toxoplasma gondii* Isolat Lokal. *Thesis*. Program Studi Bioteknologi Universitas Gadjah Mada, Yogyakarta.

Dubey JP. 2007. The History and Life Cycle of *Toxoplasma gondii*. In: *Toxoplasma gondii.* The Model Apicomplexan: Perspective and Methods. Weiss LM and Kim K. Eds. Elsevier Ltd. Great Britain. Pp. 1-12.

Gandahusada S. 1995. Penanggulangan Toksoplasmosis dalam Meningkatkan Kualitas Sumber Daya Manusia. Majalah Kedokteran Indonesia 45(6):365-370.

Garcia- Réquet N, M Lebrun, M Fourmaux, O Mercereau-Puijalon, Taramann, JM Beckers, B Samyn, J Van Beeumen, D Bout and J Dubremetz . 2000. The microneme protein MIC3 of *Toxoplasma gondii* is a secretory adhesin that binds to both the surface of the host cells and the surface of the parasit. Cellular Microbiol. 2(4):353-364.

Hiszczyńska-Sawicka E, J Kur, H Pietkiewicz, L Holec, A Gasior and P Myjak. 2005. Efficient production of the *Toxoplasma gondii* GRA6, p35, SAG2, recombinant antigens and their applications in the serodiagnosis of toxoplasmosis. Acta Parasitologica 50(3) 249-254.

Igarashi M, F Kano, K Tamekuni, PM Kawasaki, LT Navarro, O Vidotto, MC Vidotto, RZ Machado

and JL Garcia. 2008. *Toxoplasma gondii :* Cloning, sequencing, expression, and antigenic characterization of ROP2, GRA5, and GRA7. Genetics and Molecular Res. 7(2):305-313.

Indrasanti D, A Haryanto and WT Artama. 2011. Subkloning dan isolasi gen penyandi mikronema 3 (MIC3) toxoplasma gondii isolat lokal. J. Ilmiah Kimia Molekul 6(1):10-18.

Ismael AB, D Sekkai, C Collin, D Bout and M Mévélec . 2003. The MIC3 Gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. Infection and Immunity. 71(11):6222-6228.

Novagen. 2006. pET system manual. 11th ed. www.novagen.com. (Accesed: 17th February 2008).

Tenter AM. 2009. *Toxoplasma gondii* in animals used for human consumption. Mem Inst Oswaldo Cruz. 104(2):364-369.

Tenter AM, AR Heckeroth and LM Weiss. 2000. *Toxoplasma gondii*: from animals to human. Int. J. Parasitol. 30:1217-1258.

Wihadmadyatami H. 2009. Kajian Serologis Toksoplasmosis pada Sapi dengan Metode *Enzyme Linked Immunosorbent Assay* Menggunakan Protein Rekombinan GRA1. *Thesis*. Program Studi Sains Veteriner. Universitas Gadjah Mada. Yogyakarta.